

UNITED STATES CONTINUING UTILITY PATENT APPLICATION
under 37 C.F.R. § 1.53(b)

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Atty. Docket No. 12441.00002

Assistant Commissioner of Patents
Box Patent Applications
Washington, D.C. 20231

Enclosed herewith is a continuing patent application and the following papers:

First Named Inventor (or application identifier): MICHAEL A. INNIS

Title of Invention: PRODUCTION OF TISSUE FACTOR PATHWAY INHIBITOR

- ☒ Continuation
☐ Divisional
☐ Continuation-in-Part

of prior application No. 08/854,764, filed May 12, 1997

1. ☒ Specification 31 pages (including specification, claims, abstract) / 11 claims (1 independent)
2. ☒ Declaration/Power of Attorney:
 - ☒ Copy from Prior Application (for continuation or divisional application)
 - ☐ Newly Executed Declaration (for CIP application)
 - ☐ Deferred under 37 C.F.R. § 1.53(f)
 - ☐ Deletion of Inventor(s) - Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b)
 - ☒ Incorporation by Reference - The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein
3. ☒ 4 Distinct sheets of ☒ Formal ☐ Informal Drawings
4. ☐ Preliminary Amendment
5. ☐ Information Disclosure Statement
 - ☐ Form 1449
 - ☐ A copy of each cited prior art reference
6. ☒ Assignment
 - ☐ Assignment with Cover Sheet attached
 - ☒ Assignment filed in prior application. Application assigned to: **Chiron Corporation**
7. ☒ Priority is hereby claimed under 35 U.S.C. § 120 based upon the following application(s):

JC862 U.S. PTO
09/639273
08/15/00

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Country	Application Number	Date of Filing (day, month, year)
US	08/854,764	12 May 1997
US	08/286,530	05 August 1994

8. ☐ Priority Document(s)
- ☐ was filed in prior application
- ☐ is attached
- ☐ will be submitted prior to or concurrently with the payment of any issue fee for the instant application

9. ☐ Small Entity Statement
- ☐ Small Entity Statement was filed in prior application, Small Entity Status is still proper and desired
- ☐ is attached
- ☐ is no longer claimed

10. ☐ Microfiche Computer Program (Appendix)

11. ☐ Nucleotide and/or Amino Acid Sequence Submission
- ☐ Computer Readable Copy
- ☐ Paper Copy (identical to computer copy)
- ☐ Statement verifying identity of above copies

12. Calculation of Fees:

FEES FOR	EXCESS CLAIMS	FEE	AMOUNT DUE
Basic Filing Fee (37 C.F.R. § 1.16(a))			\$690.00
Total Claims in Excess of 20 (37 C.F.R. § 1.16(c))	0	18.00	\$360.00
Independent Claims in Excess of 3 (37 C.F.R. § 1.16(b))	0	78.00	\$234.00
Multiple Dependent Claims (37 C.F.R. § 1.16(d))	0	260.00	\$2,600.00
Subtotal - Filing Fee Due			\$3,884.00
	MULTIPLY BY		
Reduction by 50%, if Small Entity (37 C.F.R. §§ 1.9, 1.27, 1.28)	0		\$1,942.00
TOTAL FILING FEE DUE			\$1,942.00
Assignment Recordation Fee (if applicable) (37 C.F.R. § 1.21(h))	0	40.00	\$400.00
GRAND TOTAL DUE			\$2,342.00

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13. PAYMENT is:

- ☐ included in the amount of the GRAND TOTAL by our enclosed check. A general authorization under 37 C.F.R. § 1.25(b), second sentence, is hereby given to credit or debit our Deposit Account No. 19-0733 for the instant filing and for any other fees during the pendency of this application under 37 C.F.R. §§ 1.16, 1.17 and 1.18
- ☒ not included, but deferred under 37 C.F.R. § 1.53(f).

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PRODUCTION OF TISSUE FACTOR PATHWAY INHIBITOR

BACKGROUND OF THE INVENTION

The present invention relates to a method for the production of factor VIIa/TF/Xa binding proteins, more specifically to the production of Tissue Factor Pathway Inhibitor (TFPI) and Tissue Factor Pathway Inhibitor 2 (TFPI-2), and muteins thereof in yeast cells and isolation of such polypeptides from within the yeast cell.

Tissue Factor Pathway Inhibitor (TFPI) inhibits the coagulation cascade in at least two ways: preventing formation of factor VIIa/tissue factor complex and by binding to the active site of factor Xa. The primary sequence of TFPI, deduced from cDNA sequence, indicates that the protein contains three Kunitz-type enzyme inhibitor domains. The first of these domains is required for the inhibition of the factor VIIa/tissue factor complex. The second Kunitz-type domain is needed for the inhibition of factor Xa. The function of the third Kunitz-type domain is unknown. TFPI has no known enzymatic activity and is thought to inhibit its protease targets in a stoichiometric manner; namely, binding of one TFPI Kunitz-type domain to the active site of one protease molecule. The carboxy-terminal end of TFPI is believed to have a role in cell surface localization via heparin binding and by interaction with phospholipid. TFPI is also known as Lipoprotein Associated Coagulation Inhibitor (LACI), Tissue Factor Inhibitor (TFI), and Extrinsic Pathway Inhibitor (EPI).

Mature TFPI is 276 amino acids in length with a negatively charged amino terminal end and a positively charged carboxy-terminal end. TFPI contains 18 cysteine residues and forms 9 disulphide bridges when correctly folded. The primary sequence also contains three Asn-X-Ser/Thr N-linked glycosylation consensus sites, the asparagine residues located at positions 145, 195 and 256. The carbohydrate component of mature TFPI is approximately 30% of the mass of the protein. However, data from proteolytic mapping and mass spectral data imply that the carbohydrate moieties are heterogeneous. TFPI is also found to be phosphorylated at the serine residue in position 2 of the protein to varying degrees. The phosphorylation does not appear to affect TFPI function.

TFPI has been shown to prevent mortality in a lethal *Escherichia coli* (*E. coli*) septic shock baboon model. Creasey *et al*, J. Clin. Invest. 91:2850-2860 (1993). Administration of TFPI at 6 mg/kg body weight shortly after infusion of a lethal dose of *E. coli* resulted in survival in all five TFPI-treated animals with significant improvement in quality of life compared with a mean survival time for the five control animals of 39.9 hours. The administration of TFPI also resulted in significant attenuation of the coagulation response, of various measures of cell injury and significant reduction in pathology normally observed in *E. coli* sepsis target organs, including kidneys, adrenal glands, and lungs.

Due to its clot-inhibiting properties, TFPI may also be used to prevent thrombosis during microvascular surgery. For example, U.S. 5,276,015 discloses the use of TFPI in a method for reducing thrombogenicity of microvascular anastomoses wherein TFPI is administered at the site of the microvascular anastomoses contemporaneously with microvascular reconstruction.

Recently, another protein with a high degree of structural identity to TFPI has been identified. Sprecher *et al*, Proc. Nat. Acad. Sci., USA 91:3353-3357 (1994). The predicted secondary structure of this protein, called TFPI-2, is virtually identical to TFPI with 3 Kunitz-type domains, 9 cysteine-cysteine linkages, an acidic amino terminus and a basic carboxy-terminal tail. The three Kunitz-type domains of TFPI-2 exhibit 43%, 35% and 53% primary sequence identity with TFPI Kunitz-type domains 1, 2, and 3, respectively. Recombinant TFPI-2 strongly inhibits the amidolytic activity of factor VIIa/tissue factor. By contrast, TFPI-2 is a weak inhibitor of factor Xa amidolytic activity.

TFPI has been isolated from human plasma and from human tissue culture cells including HepG2, Chang liver and SK hepatoma cells. Recombinant TFPI has been expressed in mouse C127 cells, baby hamster kidney cells, Chinese hamster ovary cells and human SK hepatoma cells. Recombinant TFPI from the mouse C127 cells has been shown in animal models to inhibit tissue-factor induced coagulation. Frequently, however, TFPI produced in mammalian cells is degraded by proteolytic cleavage. The protein is most often cleaved at the arginine at positions 83 and 199.

A non-glycosylated form of recombinant TFPI has been produced and isolated from

Escherichia coli (*E. coli*) cells as disclosed in U.S. Pat. No. 5,212,091. This form of TFPI has been shown to be active in the inhibition of bovine factor Xa and in the inhibition of human tissue factor-induced coagulation in plasma. In some assays, the *E. coli*-produced TFPI has been shown to be more active than TFPI derived from SK hepatoma cells. However, TFPI produced in *E. coli* cells is frequently modified in ways that increase heterogeneity of the protein. These modifications include proteolytic degradation, carbamylation, and N-terminal modifications. Different forms of TFPI may also be found resulting from inappropriate internal translation initiation by *E. coli* ribosomes.

Methods have also been disclosed for purification of TFPI from yeast cell culture medium, such as in Petersen *et al*, J.Biol.Chem. 18:13344-13351 (1993). In these cases, recombinant TFPI is secreted from the yeast cell. TFPI recovered in such protocols is also frequently heterogeneous due perhaps to proteolytic degradation and variable glycosylation. Therefore, a need exists in the art to produce mature TFPI that is authentic (i.e. having the correct N-terminal amino acid sequence), full-length and homogeneous.

SUMMARY OF THE INVENTION

The invention relates to a method for producing factor VIIa/TF/Xa binding proteins, including TFPI, TFPI-2, and muteins thereof, the method including the steps of incubating yeast cells transformed with a replicable cloning vehicle, the replicable cloning vehicle comprising a first nucleotide sequence encoding the factor VIIa/TF/Xa binding protein, under conditions favorable for production of the factor VIIa/TF/Xa binding protein, wherein the factor VIIa/TF/Xa binding protein is retained within the yeast cell, preparing an insoluble fraction of the transformed yeast cells containing the factor VIIa/TF/Xa binding protein, and isolating the factor VIIa/TF/Xa binding protein contained in the insoluble fraction. The DNA encoding the factor VIIa/TF/Xa binding protein may be immediately preceded in frame by a second nucleotide sequence, the first and said second nucleotide sequences together encoding a fusion peptide, the fusion peptide capable of being cleaved within the yeast cells to produce authentic factor VIIa/TF/Xa binding protein. The second nucleotide sequence may encode ubiquitin. The yeast

cells may be from the genus *Saccharomyces*, particularly *Saccharomyces cerevisiae* and may have a genotype selected from the group consisting of: VH6, AB122, and JSC310.

The invention also relates to the factor VIIa/TF/Xa binding protein produced by the method of the invention. Where the factor VIIa/TF/Xa binding protein is a mutein of TFPI, the mutein of TFPI may have an arginine in the P₁-reactive site of Kunitz-type domain 1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and 1B show the activity of TFPI produced by the method of the invention.

Figure 2 depicts a schematic of a replicable cloning vehicle (designated pLACI 4.1) including a DNA sequence coding for the TFPI protein.

Figure 3 depicts sequence encoding a ubiquitin-TFPI fusion protein [SEQ ID NO: 1] used to produce TFPI containing the authentic primary sequence in the method of the invention.

Figure 4 shows a Coomassie stained PAGE gel of TFPI contained in an insoluble fraction within yeast cells after an initial purification.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "factor VIIa/TF/Xa binding protein" refers to proteins capable of binding to the factor VIIa/TF complex thereby inhibiting the function of the complex and further capable of binding factor Xa thereby inhibiting its function. Full-length mature TFPI, TFPI-2 and muteins thereof are encompassed by this term. Preferably, the factor VIIa/TF/Xa binding protein is TFPI.

It has now been found that production of factor VIIa/TF/Xa binding proteins, specifically TFPI in yeast, wherein the TFPI is not secreted but is retained within the cells in an insoluble fraction, leads to recovery of authentic (i.e. having the correct N-terminal amino acid sequence), full-length and homogeneous TFPI. As used herein, the term "TFPI" refers to the 276 amino acid polypeptide as described in Girard *et al*, Nature, 338:518-520 (1989). As used herein, TFPI-2 refers to the 213 amino acid polypeptide as described in Sprecher *et al*, Proc. Nat. Acad. Sci., USA 91:3353-3357 (1994).

Yeast expression systems that can be used in the present invention for the production of factor VIIa/TF/Xa binding protein are known to one of ordinary skill in the art. Such expression systems require, at a minimum, a yeast promoter which is a DNA sequence that is capable of binding yeast RNA polymerase and initiating the downstream (5'-3') transcription of a coding sequence (e.g. DNA encoding TFPI or TFPI-2) into mRNA. The promoter herein will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site (the "TATA box") and a transcription initiation site. A yeast promoter herein may also have a second domain called an upstream activator sequence (UAS), which, if present, is usually distal to the structural gene (i.e. further upstream) relative to the transcription initiation region. The UAS also governs regulation of expression. Regulated expression may be either positive or negative, thereby either enhancing or reducing transcription as desired.

Yeast is a fermenting organism with an active metabolic pathway, therefore sequences encoding enzymes in the metabolic pathway provide particularly useful promoter sequences for the purpose of the present invention. Examples include alcohol dehydrogenase (ADH) (EPO Publ. No. 284 044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EPO Publ. No. 329 203). The yeast PHO5 gene, encoding acid phosphatase and described in Myanohara *et al* Proc. Natl. Acad. Sci. USA 80:1 (1983), also provides useful promoter sequences for the present invention.

In addition, synthetic promoters which do not occur in nature also function as yeast promoters herein. For example, UAS sequences of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region as described in U.S. Patent Nos. 4,876,197 and 4,880,734, the disclosures of which are herein incorporated by reference. Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the ADH2, GAL4, GAL10, OR PHO5 genes, combined with the transcriptional activation region of a glycolytic enzyme gene

such as GAP or PyK, as described in EPO Publ. No. 164 556. Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription. Examples of such promoters include those described in the following references: Cohen *et al*, Proc. Natl. Acad. Sci. USA 77:1078 (1980); Henikoff *et al*,
5 Nature 283:835 (1981); Hollenberg *et al*, Curr. Topics Microbiol. Immunol. (1981) 96:119;
Hollenberg *et al*, "The Expression of Bacterial Antibiotic Resistance Genes in the Yeast
Saccharomyces cerevisiae," in: Plasmids of Medical, Environmental and Commercial
Importance (eds. K.N. Timmis and A. Puhler) (1979); Mercerau-Puigalon *et al*, (1980) Gene
11:163; Panthier *et al*, Curr. Genet. 2:109 (1980).

10 The production of fusion proteins in a yeast expression system is preferred for the
purpose of the present invention. More specifically, a DNA sequence encoding the N-terminal
portion of an endogenous yeast protein, or other stable protein, is fused to the 5' end of the factor
VIIa/TF/Xa binding protein encoding sequences. Upon expression, this construct will provide a
fusion of the two amino acid sequences. For example, the yeast or human superoxide dismutase
15 (SOD) gene, can be linked at the 5' terminus of the TFPI gene and the resulting fusion protein
expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or
may not encode a cleavable site as described in EPO Publ. No. 196 056. Preferably, the site is
cleavable. A preferred fusion protein is a ubiquitin-TFPI fusion protein. Such a fusion protein is
made with the ubiquitin sequence that preferably retains a site for a processing enzyme which
20 allows a ubiquitin-specific processing protease to cleave the ubiquitin from the desired
polypeptide. Through this method, therefore, mature factor VIIa/TF/Xa binding protein having
an authentic amino terminus can be produced in and isolated from within the yeast cell. Use of
the ubiquitin protein fusion technique is reviewed in Barr *et al*, in RECOMBINANT SYSTEMS
IN PROTEIN EXPRESSION (Elsevier Science Publishers B.V., 1991), pp. 37-46.

25 Usually, transcription termination sequences recognized by yeast are regulatory regions
located 3' to the translation stop codon and, together with the promoter, flank the coding
sequence. These sequences direct the transcription of an mRNA which can be translated into the
polypeptide encoded by the DNA. Yeast-recognized termination sequences from genes, such as

those coding for alpha-factor and for glycolytic enzymes, can be used herein.

Usually, the above described components, comprising a promoter, optionally a leader, the factor VIIa/TF/Xa binding protein encoding sequence, and transcription termination sequence, are put together into expression constructs. Expression constructs herein can be maintained in a replicon, such as an extrachromosomal element (e.g., a plasmid) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing the replicon to be maintained, for example, in yeast for expression and in a procaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors that are useful herein include YEp24 as described in Botstein *et al*, (1979) Gene 8:17-24; pCI/1, as described in Brake *et al*, (1984) Proc. Natl. Acad. Sci USA 81:4642-4646; and YRp17, as described in Stinchcomb *et al*, (1982) J. Mol. Biol. 158:157. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host. See e.g., Brake *et al*, *supra*.

Alternatively, the expression constructs can be integrated into the yeast genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to result from recombinations between homologous DNA in the vector and the yeast chromosome. An integrating vector may be directed to a specific locus in yeast by selecting the appropriate homologous sequence for inclusion in the vector, as described in Orr-Weaver *et al*, Methods in Enzymol. 101:228-245 (1983). One or more expression construct may integrate, possibly affecting levels of recombinant protein produced. Rine *et al*, Proc. Natl. Acad. Sci. USA 80:6750 (1983). The chromosomal sequences included in the vector can occur either as a single segment in the vector, which results in the integration of the entire vector, or two segments homologous to adjacent segments in the chromosome and flanking the expression construct in the vector, which can result in the stable integration of only the expression construct.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of yeast strains that have been transformed. Selectable markers may include biosynthetic genes that can be expressed in the yeast host, such as ADE2, HIS4, LEU2, TRP1, and ALG7, and the G418 resistance gene, which confer resistance in yeast cells to tunicamycin and G418, respectively. In addition, a suitable selectable marker may also provide yeast with the ability to grow in the presence of toxic compounds, such as metal. For example, the presence of CUP1 allows yeast to grow in the presence of copper ions as described in Butt *et al*, Microbiol. Rev. 51:351 (1987).

Alternatively, the above described components can be put together into transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, inter alia, the following yeasts: *Candida albicans* (Kurtz, *et al*, Mol. Cell. Biol. 6:142 (1986)), *Candida maltosa* (Kunze, *et al*, J. Basic Microbiol. 25:141 (1985)), *Hansenula polymorpha* (Gleeson, *et al*, J. Gen. Microbiol. 132:3459 (1986); Roggenkamp *et al*, Mol. Gen. Genet. 202:302 (1986)), *Kluyveromyces fragilis* (Das, *et al*, J. Bacteriol. 158:1165 (1984)), *Kluyveromyces lactis* (De Louvencourt *et al*, J. Bacteriol. 154:737 (1983); Van den Berg *et al*, Bio/Technology 8:135 (1990)), *Pichia guillermondii* (Kunze *et al*, J. Basic Microbiol. 25:141 (1985)), *Pichia pastoris* (Cregg, *et al*, Mol. Cell. Biol. 5:3376 (1985); U.S. Patent Nos. 4,837,148 and 4,929,555), *Saccharomyces cerevisiae* (Hinnen *et al*, Proc. Natl. Acad. Sci. USA 75:1929 (1978); Ito *et al*, J. Bacteriol. 153:163 (1983)), *Schizosaccharomyces pombe* (Beach and Nurse, Nature 300:706 (1981)), and *Yarrowia lipolytica* (Davidow, *et al*, Curr. Genet. 10:380471 (1985) and Gaillardin, *et al*, Curr. Genet. 10:49 (1985)).

Transformation procedures that may be used herein to transform yeast cells include electroporation, as described in "Guide to Yeast Genetics and Molecular Biology," Vol 194 METHODS IN ENZYMOLOGY, C. Guthrie and G.R. Fink, (Academic Press 1991). Other procedures include the transformation of spheroplasts or the transformation of alkali cation-

5 treated intact cells. Such procedures are described in, for example, Kurtz *et al*, Mol. Cell. Biol. 6:142 (1986); Kunze *et al*, J. Basic Microbiol. 25:141 (1985), for *Candida*; Gleeson *et al*, J. Gen. Microbiol. 132:3459 (1986); Roggenkamp *et al*, Mol. Gen. Genet. 202:302, for *Hansenula* (1986); Das *et al*, J. Bacteriol. 158:1165 (1984); De Louvencourt *et al*, J. Bacteriol. 154:1165 (1983); Van den Berg *et al*, Bio/Technology 8:135 (1990) for *Kluyveromyces*; Cregg *et al*, Mol. Cell. Biol. 5:3376 (1985); Kunze *et al*, J. Basic Microbiol. 25:141 (1985); U.S. Patent No. 4,837,148 and U.S. Patent No. 4,929,555, for *Pichia*; Hinnen *et al*, Proc. Natl. Acad. Sci. USA 75:1929 (1978); Ito *et al*, J. Bacteriol. 153:163 (1983), for *Saccharomyces*; Beach and Nurse Nature 300:706 (1981), for *Schizosaccharomyces*; Davidow *et al*, Curr. Genet. 10:39 (1985); Gaillardin *et al*, Curr. Genet. 10:49 (1985), for *Yarrowia*.

10 Muteins of TFPI or TFPI-2 may also be prepared according to the method of the invention. Muteins within the scope of this definition include: (a) TFPI or TFPI-2 muteins having 1-5 conservative amino acid substitutions that do not substantially change the conformation of the molecule; (b) TFPI or TFPI-2 muteins with amino acid substitutions that eliminate one or more of the sites for N-linked glycosylation; (c) TFPI muteins having 1-5 amino acid substitutions that change a residue of TFPI to a corresponding residue of TFPI-2; (d) TFPI-2 muteins having 1-5 amino acid substitutions that change a residue of TFPI-2 to a corresponding residue of TFPI; (e) TFPI or TFPI-2 muteins with amino acid substitutions in P₁-reactive sites in one or more Kunitz-type domains; and (f) TFPI or TFPI-2 muteins with amino acid substitutions at positions within 5 amino acids of the P₁-reactive sites in one or more Kunitz-type domains. In a preferred embodiment, the lysine residue in the P₁-reactive site of the first Kunitz-type domain of TFPI is replaced with arginine. The mutein has the following sequence:

25 Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu
Pro Pro Leu Lys Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp
Gly Pro Cys **Arg** Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Thr
Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn
Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn

Ala Asn Arg Ile Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe
Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg
Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly
Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys
5 Asn Ile Cys Glu Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly
Thr Gln Leu Asn Ala Val Asn Asn Ser Leu Thr Pro Gln Ser Thr Lys
Val Pro Ser Leu Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro
Ala Asp Arg Gly Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn
Ser Val Ile Gly Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly
10 Asn Glu Asn Asn Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys
Lys Gly Phe Ile Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys
Arg Lys Arg Lys Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe
Val Lys Asn Met. [SEQ ID NO: 3]

Similarly, the mutein may be a TFPI-2 mutein in which the glutamate residue in the P₁ reactive
15 site of the second Kunitz-type domain of TFPI-2 is replaced with arginine. Production and
isolation of muteins of TFPI and TFPI-2 in which one or more consensus sites for N-linked
glycosylation have been altered to prevent such recognition is also within the method of the
invention. TFPI has three such consensus sequences which may be altered for example by
substituting alanine for serine or threonine in the the consensus site. Similarly, muteins of TFPI-
20 2 in which the serine residue at position 96 of mature TFPI-2 and/or the threonine residue at
position 150 of mature TFPI-2 have been substituted with alanine may also be prepared.
Muteins containing conservative amino acid substitutions relative to the sequence of TFPI or
TFPI-2 may be produced and isolated according to the invention. Finally, production and
isolation of muteins of TFPI in which the corresponding amino acid of TFPI-2 for a given site is
25 substituted for the amino acid normally found in the TFPI sequence and muteins of TFPI-2 in
which the corresponding amino acid of TFPI for a given site is substituted for the amino acid
normally found in the TFPI sequence are within the scope of the invention.

Muteins may be prepared by appropriate mutagenesis of the sequence of the recombinant
cloning vehicle encoding TFPI or TFPI-2. Techniques for mutagenesis include, without

limitation, site specific mutagenesis. Site-specific mutagenesis can be carried out using any number of procedures known in the art. These techniques are described, for example, by Smith, 1985, Annual Review of Genetics, 19:423, and modifications of some of the techniques are described in Methods in Enzymology, 154, part E, (eds.) Wu and Grossman (1987), chapters 17, 18, 19, and 20. Site specific mutagenesis may also be carried out using the Gapped Duplex site-directed mutagenesis method. The general procedure is described by Kramer, *et al.*, in chapter 17 of the Methods in Enzymology, *supra*. Other techniques for generating point mutations in a nucleic acid sequence PCR techniques, including overlapping PCR, are described in PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, (eds.) Innis, Gelfand, Sninsky and White (Academic Press, 1990).

Formulation and Administration

LACI made by the method of the invention may be administered at a concentration that is therapeutically effective to treat and prevent septic shock. To accomplish this goal, the LACI made by the method of the invention is preferably administered intravenously. Methods to accomplish this administration are known to those of ordinary skill in the art.

Before administration to patients, formulants may be added to the LACI made by the method of the invention. A liquid formulation may be used. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents. Carbohydrates which may be used in the formulation include sugar or sugar alcohols such as mono, di, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, dextrose, lactose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, alpha and beta cyclodextrin, soluble starch, hydroxethyl starch and carboxymethylcellulose, or mixtures thereof. Sucrose is most preferred. Sugar alcohol is defined as a C₄ to C₈ hydrocarbon having an -OH group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is

no fixed limit to amount used as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v% and 7.0 w/v%, more preferable between 2.0 and 6.0 w/v%. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added.

5 Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the composition to minimize pH changes in the solution before lyophilization or after reconstitution. Most any physiological buffer may be used, but citrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Most preferred is a citrate buffer. Further, the use of sulfates should be avoided in preparation of the formulation. Preferably, the concentration is from 0.01 to 0.3 molar. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

10 Additionally, the LACI made by the method of the invention can be chemically modified by covalent conjugation to a polymer to increase its circulating half-life, for example. Preferred polymers, and methods to attach them to peptides, are shown in U.S. Patent Nos. 4,766,106, 4,179,337, 4,495,285, and 4,609,546 which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and polyethyleneglycol (PEG). PEG is soluble in water at room temperature and has the general formula:
$$R(O-CH_2-CH_2)_nO-R$$
 where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1000 and 40,000, more preferably between 2000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group.
20 It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/LACI.

Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), etc. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf et al., 1988, J. Bio. Chem. 263:15064-15070, and a discussion of POG/protein conjugates is found in U.S. Patent No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

Administration to Affected Individuals

LACI made by the method of the invention is useful to treat mammals with sepsis or septic shock. Generally, conditions are characterized by high fever ($>38.5^{\circ}\text{C}$) or hypothermia ($>35.5^{\circ}\text{C}$), low blood pressure, tachypnea (> 20 breaths/minute), tachycardia (> 100 beats/minute), leukocytosis ($> 15,000$ cells/ mm^3) and thrombocytopenia ($< 100,000$ platelets/ mm^3). The LACI made by the method of the invention is preferably administered as soon as the subject is suspected of being septic; presenting a $>20\%$ drop in fibrinogen or appearance of fibrin split products, a rise in the subject's temperature and the diagnosis of leukopenia and hypotension associated with septic shock. As stated above, intravenous administration is preferred. Generally, LACI made by the method of the invention is given at

a dose between 1 $\mu\text{g/kg}$ and 20 mg/kg , more preferably between 20 $\mu\text{g/kg}$ and 10 mg/kg , most preferably between 1 and 7 mg/kg . Preferably, it is given as a bolus dose, to increase circulating levels by 10-20 fold and for 4-6 hours after the bolus dose. Continuous infusion may also be used after the bolus dose. If so, LACI may be infused at a dose between 5 and 20 $\mu\text{g/kg/minute}$, more preferably between 7 and 15 $\mu\text{g/kg/minute}$.

The LACI made by the method of the invention may be given in combination with other agents which would be effective to treat septic shock. For example, the following may be administered in combination with the LACI made by the method of the invention: antibiotics that can treat the underlying bacterial infection; monoclonal antibodies that are directed against bacterial cell wall components; monoclonal antibodies and soluble receptors that can complex with cytokines that are involved in the sepsis pathway, including, but not limited to tumor necrosis factor (TNF), Interleukin-1, γ -interferon and interleukin-8; and generally any agent or protein that can interact with cytokines or complement proteins in the sepsis pathway to reduce their effects and to attenuate sepsis or septic shock.

Antibiotics useful in the present invention include those in the general category of: beta-lactam rings (penicillin), amino sugars in glycosidic linkage (amino glycosides), macrocyclic lactone rings (macrolides), polycyclic derivatives of naphacenecarboxamide (tetracyclines), nitrobenzene derivatives of dichloroacetic acid, peptides (bacitracin, gramicidin, and polymyxin), large rings with a conjugated double bond system (polyenes), sulfa drugs derived from sulfanilarnide (sulfonamides), 5-nitro-2-furanyl groups (nitrofurans), quinolone carboxylic acids (nalidixic acid), and many others. Other antibiotics and more versions of the above specific antibiotics may be found in Encyclopedia of Chemical Technology, 3rd Edition, Kirk-Othmer (ed.), Vol. 2, pages 782-1036 (1978) and Vol. 3, pages 1-78, Zinsser, MicroBiology, 17th Edition W. Joklik *et al* (Eds.) pages 235-277 (1980), or Dorland's Illustrated Medical Dictionary, 27th Edition, W.B. Saunders Company (1988).

Other agents which may be combined with the LACI made by the method of the invention include monoclonal antibodies directed to cytokines involved in the sepsis pathway,

such as those monoclonal antibodies directed to IL-6 or M-CSF, such as shown in PCT US90/07411; monoclonal antibodies directed to TNF, such as shown in U. S. Patent No. 4,603,106; inhibitors of proteins that cleave the mature TNF prohormone from the cell in which it was produced, such as shown in PCT US90/03266 and PCT US93/06120; antagonists of IL-1, such as shown in PCT US91/02460; inhibitors of IL-6 cytokine action such as activin, such as shown in PCT US90/00321; and receptor based inhibitors of various cytokine such as IL-1. Antibodies to or small molecule inhibitors of complement protein may also be employed.

Generally, the LACI made by the method of the invention may be useful for those diseases that occur due to the up-regulation of tissue factor brought on by injury, trauma, endotoxin, TNF, cancer, IL-1 or other agents or conditions.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

EXAMPLES

Example 1

The shuttle vector pBS24 is described in Barr *et al*, EXPRESSION SYSTEMS & PROCESSES FOR rDNA PRODUCTS (American Chemical Society, 1991), pp 51-64). pBS24Ub is a derivative of pBS24.1, and contains an expression cassette flanked by unique *Bam* HI and *Sal* I restriction sites, the glucose regulatable ADH2/GAP promoter and a synthetic ubiquitin (Ub) gene. For construction of Ub fusions, a unique *Sst*II site is generated in the 3' end of the Ub gene. The presence of the *Sst*II site allows in-frame insertion of nucleotide sequences for expression as ubiquitin fusion peptides. Insertion can be accomplished by use of synthetic DNA adapters or PCR methodologies. In either case, the 5'-junction sequence will be:

ARG GLY GLY
C CGC GGY GGC
G GCG CCA CCG
*Sst*II

[SEQ ID NO: 4]

and the 3' cloning site (*Sal* I) should be as close as possible to the 3' end of the termination codon.

5 PCR was used to construct the ubiquitin/TFPI gene fusion in the 15.4kb plasmid pLACI 4.1 shown in Figure 3. TFPI encoding nucleic acid was amplified using standard PCR procedures with the primers LACI4 and LACI3. LACI4 hybridizes to the 10 nucleotides at the 5' end of nucleic acid mature encoding TFPI and also contains ubiquitin sequence with the *Sst*II restriction site. LACI3 hybridizes to the 15 nucleotides at the 3' end of nucleic acid encoding mature TFPI and also trailing sequence with a *Sal* I restriction site. The sequences of these primers are as follows:

LACI4 GCTCCGCGGTGGCGATTCTGAGG [SEQ ID NO: 5]

LACI3 TCTGTCGACTCACATATTTTAAAC [SEQ ID NO: 6]

15 After amplification, the PCR product was digested with *Sal* I and *Sst* II using conditions specified by the manufacturer of the enzymes. The digested PCR product was then cloned into pBS24Ub, as described above, to produce pLACI 4.1.

pLACI 4.1 was used to transform three strains of *Saccharomyces cerevisiae*: VH6 (MAT α , *cir*^o, *leu*-2-112,-3, *ura*3, *FoA*, *pep4::His*3), AB122 (MAT α , *cir*^o, *leu*2, *ura*3-52, *prb*1-1112, *pep*4-3, *prc*1-407) and JSC310 (as AB122, + *ADR*1 overexpression). Transformants of VH6 produced TFPI at levels of approximately 5% of total protein, transformants of AB122 produced TFPI at levels of approximately 10% of total protein and transformants of JSC310 produced TFPI at levels of approximately 15% of total protein. The stability of TFPI is somewhat surprising in view of prior studies showing that proteins synthesized as ubiquitin fusions and having N-terminal aspartate after removal of the ubiquitin are unstable in the yeast cell (e.g. half-life < 3 min.). See Finley, "The Yeast Ubiquitin System" in: The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Gene Expression. (1992).

Example 2

Part A

TFPI was purified from *S. cerevisiae* VH6 cells according to the method disclosed in U.S. Pat. No. 5,212,091, which is herein incorporated by reference, specifically at columns 9-10. An insoluble fraction (also referred to as a membrane fraction) was prepared by Dyno-Mill glass bead extraction in TEN (50 mM Tris.HCl pH7.8; 50 mM NaCl; 5 mM EDTA). Approximately 3
5 μ g of total protein from this fraction was run out on 14% SDS-PAGE gel and transferred to nitrocellulose membranes. The nitrocellulose blots were probed with rabbit polyclonal antisera generated to the first fifteen amino acids of the mature TFPI peptide sequence. The resulting blot (Figure 5) shows a significant band of homogeneous, full-length TFPI. Expression of TFPI into the insoluble fraction was also verified using comparative Coomassie-stained 14% SDS-PAGE gels. (See Figure 4). Yeast cells were grown with plasmid-containing DNA encoding the TFPI protein, or with the identical plasmid without DNA encoding the TFPI protein. Approximately 10 μ g of yeast whole cell lysate or insoluble fraction from the two cultures was loaded onto the gel. A unique 36 kD band was detected only in the lanes prepared from cultures carrying the TFPI-producing plasmid.

15 Sulfonation and Q Sepharose® fractionation were performed as specified in U.S. 5,212,091. Refolding of the isolated TFPI was also performed essentially as described except that dialysis was performed in 5 M urea, 0.3 M NaCl, 20 mM Tris.HCl pH 7.8, 0.04% NP40, 2 mM L-cysteine, 0.5 mM EDTA for 4-6 at room temperature and then 4° C overnight, after which dialysis bags were transferred to 2 M urea, 1 mM L-cysteine for 48 hours at 4° C. For S
20 Sepharose® fractionation, the refolded TFPI was equilibrated in 2 M urea, 50 mM NaPO₄, 0.1 M NaCl prior to column loading. Wash buffer contained 2 M urea, 50 mM NaPO₄, 0.25 M NaCl and TFPI was step-eluted with buffer containing 2 M urea, 50 mM NaPO₄, 0.8 M NaCl. Fractions containing TFPI were pooled and dialyzed against PBS/1.5 M urea at 4° C overnight.

25 N-terminal sequencing of the product recovered by this method gave the following correct sequence:

Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu Pro Pro [SEQ ID

NO: 6]

corresponding to authentic mature TFPI at >90% purity.

The recovered product was also tested in the factor Xa amidolytic assay and prothrombin time clotting assays (both described in Wun *et al*, J. Biol. Chem. 265:16096 (1990)) to assess activity. The data from these assays are displayed in Figures 1A and 1B, respectively. PBS buffer was used as a control in the factor Xa Inhibition Assay and PBS buffer as well as normal human serum (NHS) were used as controls in the prothrombin clotting assay. The yeast-produced TFPI inhibited factor Xa activity at a 50% inhibitory concentration of approximately 20 ng/ml and displayed an inhibitory concentration in the prothrombin clotting assay of 1µg/ml. These results indicate that TFPI produced according to the method of the invention is biologically active.

Example 3

The method of the invention can also be used to produce TFPI in large scale, i.e. 10 liter or greater batches. Yeast strain AB122 was transformed with pLACI 4.1 and grown in selective medium containing Yeast Nitrogen Base without amino acids (Difco), supplemented with 87 mg/L adenine, 43.5mg/L L-tryptophan, 43.5mg/L L-histidine, 43.5mg/L L-arginine, 43.5 mg/L L-methionine, 65.2mg/L L-tyrosine, 109mg/L L-phenylalanine, 65.2mg/L L-lysine and 8% glucose. Aliquots of the transformed cells were preserved by adding glycerol to 15% and storage at -70°C. *S. cerevisiae* strain AB122 transformed with pLACI4.1 has been deposited with the ATCC on July 19, 1994 and has been given Accession Number 74291.

Inoculum for the 10 liter fermenter was prepared by addition of 1% v/v frozen, transformed culture into fresh selective media as described in the previous paragraph. The inoculum was then grown in shake flasks for 24-48 hours. Inoculum was then added 0.5% v/v to the contents of a 10 liter fermentation vessel. The media in the vessel contained the following ingredients prior to sterilization:

Tastone 154 Yeast Extract: 10 g/L
Casein Peptone: 20 g/L
Antifoam: 0.3 ml/L
Citric acid: 4 mM

KH_2PO_4 : 20 mM

$(\text{NH}_4)_2\text{SO}_4$: 50 mM

MnSO_4 : 20 μM

ZnSO_4 : 20 μM

5 H_3BO_3 : 100 μM

CoCl_2 : 10 μM

Na_2MoO_4 : 10 μM

CuSO_4 : 2 μM

Glycerol: 30 g/L.

10 The contents of the fermentation vessel were then sterilized in situ and the following sterile ingredients added to complete the medium:

MgSO_4 : 20 mM

FeCl_3 : 100 μM

Glucose: 20g/L

15 Pyroxidine HCl: 5 mg/L

Thiamine HCl: 10mg/L

D-Biotin: 0.1 mg/L

Ca Pantothenate: 10 mg/L

Myo-Inositol: 400 mg/L.

20 During growth of cells in the fermentation vessel, the medium was maintained at a temperature of 30°C. pH 6.0 was maintained by addition of NaOH or phosphoric acid as needed. Agitation was begun at 600 r.p.m. and increased as needed to maintain D.O > 30%. Aeration was 1 vvm air. Starting at 15 h after inoculation, a mixture of sterile glucose (25% w/v) and glycerol (6.8% w/v) was added to the fermentor at a rate of 0.1 ml/min/L. At 39 hours,
25 the rate of addition of this mixture was decreased to 0.05 ml/min/L. The culture was terminated at 96 hours. Peak expression of TFPI (1 mg/ml) occurred by 72 h post-inoculation.

The foregoing discussion and examples only illustrate the present invention, persons of

	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
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20

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Innis, Michael
Creasey, Abba

(ii) TITLE OF INVENTION: Production of Tissue Factor Pathway
Inhibitor

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Chiron Corporation
(B) STREET: 4560 Horton St.
(C) CITY: Emeryville
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94608

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30B

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE: 05-AUG-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Savereide, Paul B.
(B) REGISTRATION NUMBER: 36,914
(C) REFERENCE/DOCKET NUMBER: 0991.001

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 510-601-2585

(B) TELEFAX: 510-655-3542

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1065 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 1..1056

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG CAG ATC TTC GTC AAG ACT TTG ACC GGT AAA ACC ATA ACA TTG GAA 48
Met Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Thr Leu Glu
1 5 10 15
GTT GAA TCT TCC GAT ACC ATC GAC AAC GTT AAG TCG AAA ATT CAA GAC 96
Val Glu Ser Ser Asp Thr Ile Asp Asn Val Lys Ser Lys Ile Gln Asp
20 25 30
AAG GAA GGT ATC CCT CCA GAT CAA CAA AGA TTG ATC TTT GCC GGT AAG 144
Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys
35 40 45
CAG CTA GAA GAC GGT AGA ACG CTG TCT GAT TAC AAC ATT CAG AAG GAG 192
Gln Leu Glu Asp Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu

PATENT
Atty. Docket No. 991.001

	50	55	60	
	TCC ACC TTA CAT CTT GTG CTA AGG CTC CGC GGT GGT	GAT TCT GAG GAA	240	
5	Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly	Asp Ser Glu Glu		
	65	70	75	80
	GAT GAA GAA CAC ACA ATT ATC ACA GAT ACG GAG TTG CCA CCA CTG AAA		288	
	Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu Pro Pro Leu Lys			
		85	90	95
10	CTT ATG CAT TCA TTT TGT GCA TTC AAG GCG GAT GAT GGC CCA TGT AAA		336	
	Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys			
		100	105	110
15	GCA ATC ATG AAA AGA TTT TTC TTC AAT ATT TTC ACT CGA CAG TGC GAA		384	
	Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu			
		115	120	125
20	GAA TTT ATA TAT GGG GGA TGT GAA GGA AAT CAG AAT CGA TTT GAA AGT		432	
	Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn Arg Phe Glu Ser			
		130	135	140
25	CTG GAA GAG TGC AAA AAA ATG TGT ACA AGA GAT AAT GCA AAC AGG ATT		480	
	Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn Ala Asn Arg Ile			
		145	150	155
30	ATA AAG ACA ACA TTG CAA CAA GAA AAG CCA GAT TTC TGC TTT TTG GAA		528	
	Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe Cys Phe Leu Glu			
		165	170	175
35	GAA GAT CCT GGA ATA TGT CGA GGT TAT ATT ACC AGG TAT TTT TAT AAC		576	
	Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn			
		180	185	190
40	AAT CAG ACA AAA CAG TGT GAA CGT TTC AAG TAT GGT GGA TGC CTG GGC		624	
	Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu Gly			
		195	200	205
45	AAT ATG AAC AAT TTT GAG ACA CTG GAA GAA TGC AAG AAC ATT TGT GAA		672	

PATENT
Atty. Docket No. 991.001

	Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu	
	210 215 220	
5	GAT GGT CCG AAT GGT TTC CAG GTG GAT AAT TAT GGA ACC CAG CTC AAT Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly Thr Gln Leu Asn	720
	225 230 235 240	
10	GCT GTG AAT AAC TCC CTG ACT CCG CAA TCA ACC AAG GTT CCC AGC CTT Ala Val Asn Asn Ser Leu Thr Pro Gln Ser Thr Lys Val Pro Ser Leu	768
	245 250 255	
15	TTT GAA TTT CAC GGT CCC TCA TGG TGT CTC ACT CCA GCA GAC AGA GGA Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp Arg Gly	816
	260 265 270	
20	TTG TGT CGT GCC AAT GAG AAC AGA TTC TAC TAC AAT TCA GTC ATT GGG Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn Ser Val Ile Gly	864
	275 280 285	
25	AAA TGC CGC CCA TTT AAG TAC AGT GGA TGT GGG GGA AAT GAA AAC AAT Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly Asn Glu Asn Asn	912
	290 295 300	
30	TTT ACT TCC AAA CAA GAA TGT CTG AGG GCA TGT AAA AAA GGT TTC ATC Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys Gly Phe Ile	960
	305 310 315 320	
35	CAA AGA ATA TCA AAA GGA GGC CTA ATT AAA ACC AAA AGA AAA AGA AAG Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys Arg Lys Arg Lys	1008
	325 330 335	
40	AAG CAG AGA GTG AAA ATA GCA TAT GAA GAA ATT TTT GTT AAA AAT ATG Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe Val Lys Asn Met	1056
	340 345 350	
45	TGAGTCGAC	1065

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 352 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Thr Leu Glu
1 5 10 15
Val Glu Ser Ser Asp Thr Ile Asp Asn Val Lys Ser Lys Ile Gln Asp
20 25 30
Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys
35 40 45
Gln Leu Glu Asp Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu
50 55 60
Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly Asp Ser Glu Glu
65 70 75 80
Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu Pro Pro Leu Lys
85 90 95
Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys
100 105 110
Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu
115 120 125
Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn Arg Phe Glu Ser
130 135 140
Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn Ala Asn Arg Ile
145 150 155 160

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Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe Cys Phe Leu Glu
165 170 175

5 Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn
180 185 190

Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu Gly
195 200 205

10 Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu
210 215 220

Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly Thr Gln Leu Asn
225 230 235 240

15 Ala Val Asn Asn Ser Leu Thr Pro Gln Ser Thr Lys Val Pro Ser Leu
245 250 255

20 Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp Arg Gly
260 265 270

Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn Ser Val Ile Gly
275 280 285

25 Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly Asn Glu Asn Asn
290 295 300

Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys Gly Phe Ile
305 310 315 320

30 Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys Arg Lys Arg Lys
325 330 335

35 Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe Val Lys Asn Met
340 345 350

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 276 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu
1 5 10 15

Pro Pro Leu Lys Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp
20 25 30

Gly Pro Cys Arg Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Thr
35 40 45

Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn
50 55 60

Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn
65 70 75 80

Ala Asn Arg Ile Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe
85 90 95

Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg
100 105 110

Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly
115 120 125

Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys
130 135 140

Asn Ile Cys Glu Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly
145 150 155 160

Thr Gln Leu Asn Ala Val Asn Asn Ser Leu Thr Pro Gln Ser Thr Lys
165 170 175

Val Pro Ser Leu Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro
180 185 190

Ala Asp Arg Gly Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn
195 200 205

Ser Val Ile Gly Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly
210 215 220

Asn Glu Asn Asn Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys
225 230 235 240

Lys Gly Phe Ile Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys
245 250 255

Arg Lys Arg Lys Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe
260 265 270

Val Lys Asn Met
275

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCGCGGGGC

9

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTCCGCGGT GGCGATTCTG AGG

23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCTGTCGACT CACATATTTT TAAC

24

(2) INFORMATION FOR SEQ ID NO:7:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: peptide

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu
1 5 10 15

20

Pro Pro

CLAIMS

We claim:

- 5 1. A method for producing a factor VIIa/TF/Xa binding protein, comprising:
 - a) incubating yeast cells transformed with a replicable cloning vehicle, said
10 replicable cloning vehicle comprising a first nucleotide sequence encoding the
factor VIIa/TF/Xa binding protein, under conditions favorable for production of
the factor VIIa/TF/Xa binding protein, wherein the factor VIIa/TF/Xa binding
protein is retained within the yeast cell;
 - b) preparing an insoluble fraction of the transformed yeast cells containing the factor
VIIa/TF/Xa binding protein;
 - c) isolating the factor VIIa/TF/Xa binding protein of the insoluble fraction.
- 15 2. The method of claim 1 wherein the DNA encoding the factor VIIa/TF/Xa binding protein
is immediately preceded in frame by a second nucleotide sequence, said first and said second
nucleotide sequences together encoding a fusion peptide, said fusion peptide capable of being
cleaved within the yeast cells to produce authentic factor VIIa/TF/Xa binding protein.
- 20 3. The method of claim 2 wherein said second nucleotide sequence encodes ubiquitin.
4. The method of claim 3 wherein the replicable cloning vehicle comprises SEQ ID 1.
5. The method of claim 1 wherein the yeast cells are of the genus *Saccharomyces*.
- 25 6. The method of claim 5 wherein the yeast cells are of the species *Saccharomyces*
cerevisiae and have a genotype selected from the group consisting of VH6, AB122, and JSC310.

7. The factor VIIa/TF/Xa binding protein prepared by the method according to claim 1.

8. The method of claim 1 wherein the factor VIIa/TF/Xa binding protein is TFPI.

5 9. The method of claim 1 wherein the factor VIIa/TF/Xa binding protein is TFPI-2.

10. The method of claim 1 wherein the factor VIIa/TF/Xa binding protein is a mutein of TFPI having arginine in the P1 reactive site of Kunitz-type domain 1.

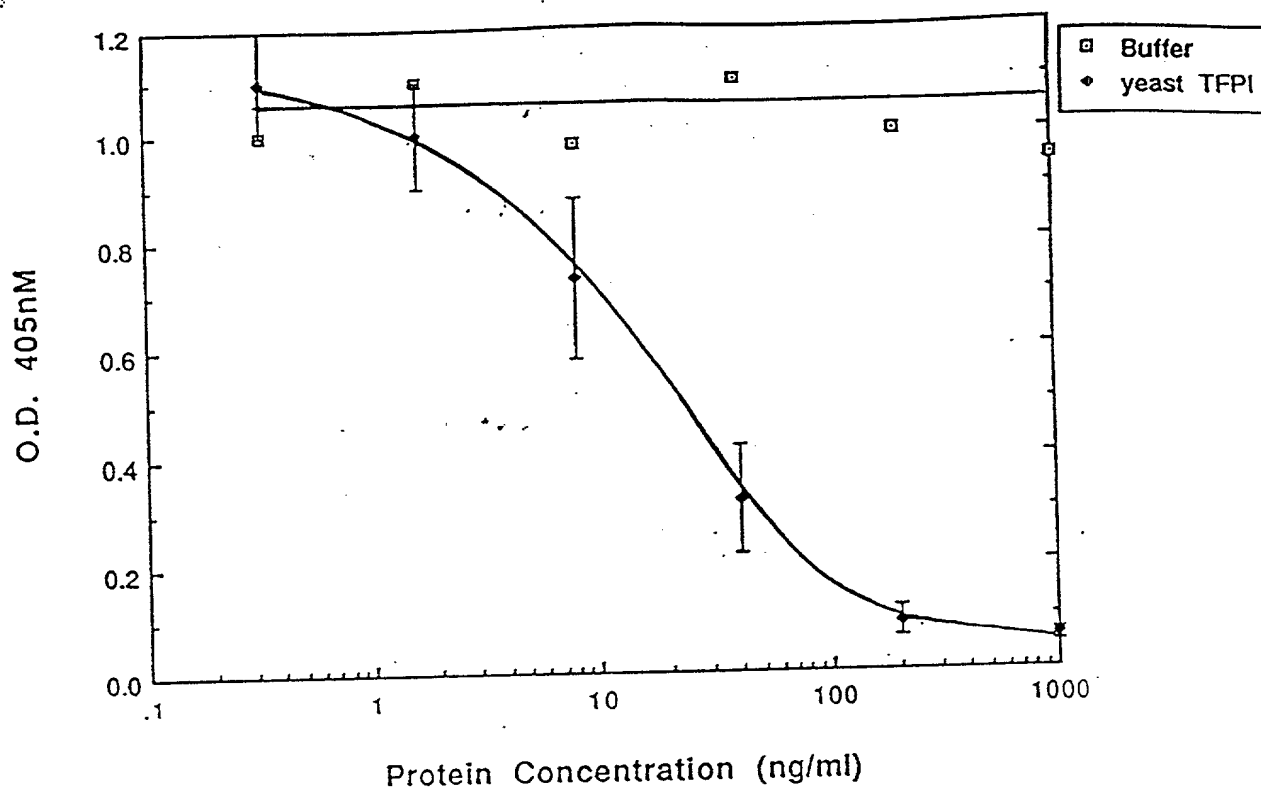
10 11. The factor VIIa/TF/Xa binding protein prepared by the method according to claim 10.

ABSTRACT OF THE DISCLOSURE

5 A method for the production of Tissue Factor Pathway Inhibitor (TFPI) and Tissue Factor Pathway Inhibitor 2 (TFPI-2), and muteins thereof is disclosed wherein the protein is retained within a yeast cell during growth of the yeast cell and recovered from an insoluble fraction prepared from yeast cells containing the protein.

Figure 1

A. Factor Xa Inhibition - Purified Yeast TFPI



B. Prothrombin Time Assay - Purified Yeast TFPI

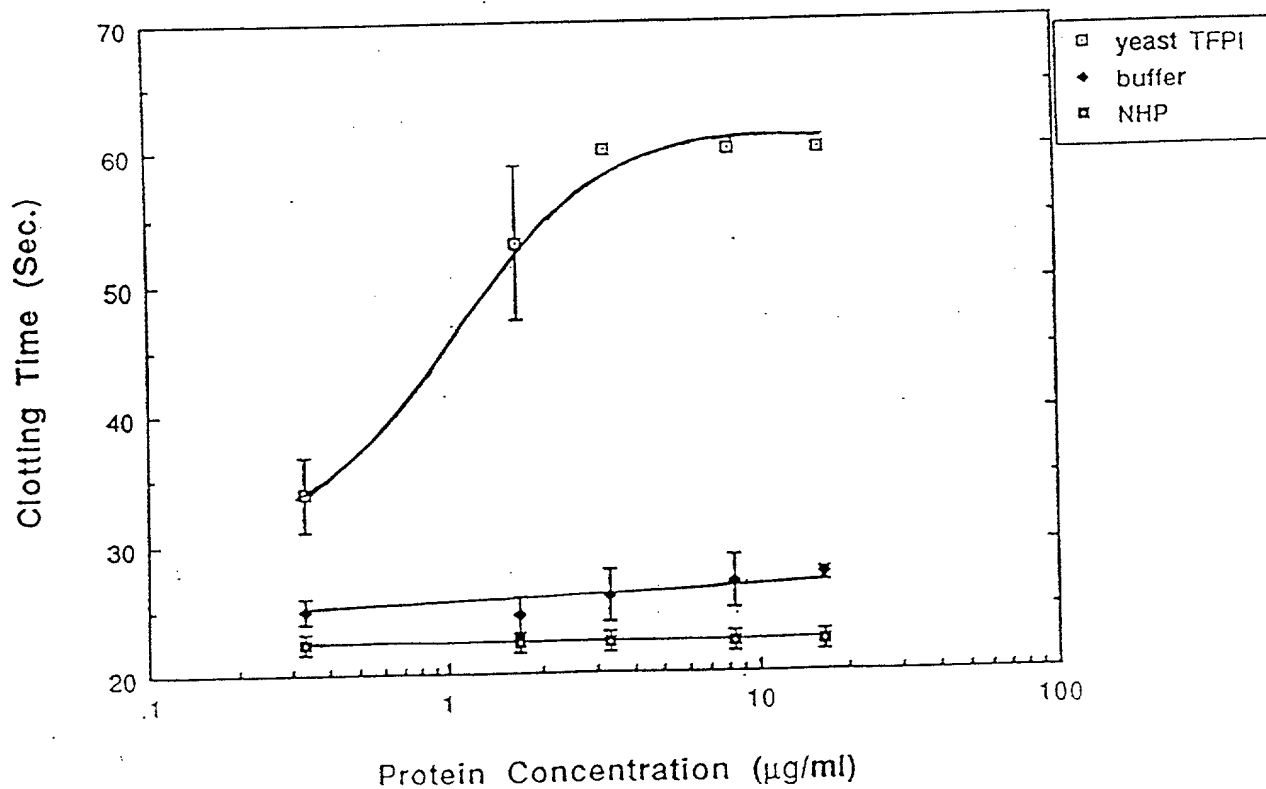


Figure 2

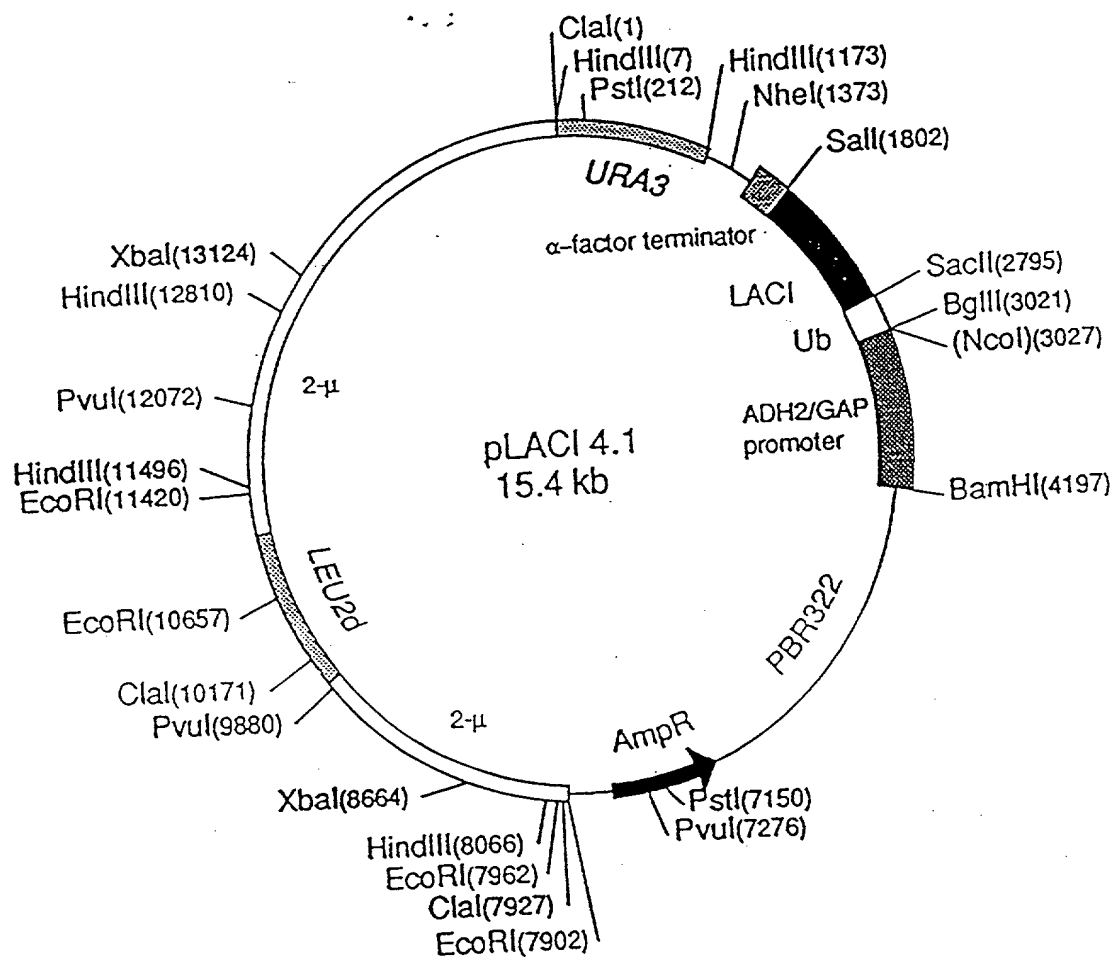
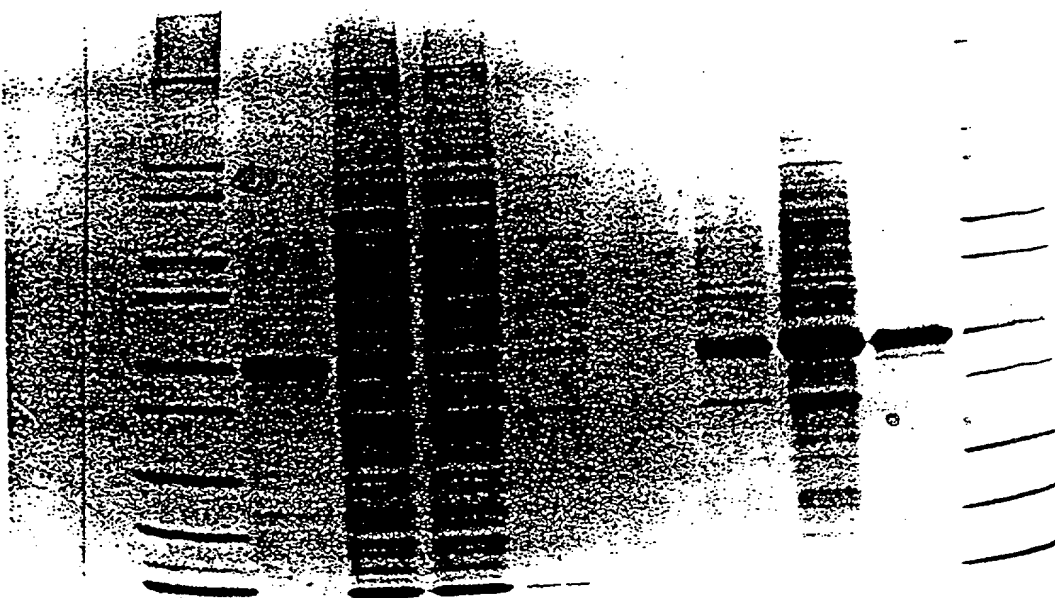


Figure 3

					5						10						15					
Met	Gln	Ile	Phe	Val	Lys	Thr	Leu	Thr	Gly	Lys	Thr	Ile	Thr	Leu	Glu							
ATG	CAG	ATC	TTC	GTC	AAG	ACT	TTG	ACC	GGT	AAA	ACC	ATA	ACA	TTG	GAA							
TAC	GTC	TAG	AAG	CAG	TTC	TGA	AAC	TGG	CCA	TTT	TGG	TAT	TGT	AAC	CTT							
					20						25						30					
Val	Glu	Ser	Ser	Asp	Thr	Ile	Asp	Asn	Val	Lys	Ser	Lys	Ile	Gln	Asp							
GTT	GAA	TCT	TCC	GAT	ACC	ATC	GAC	AAC	GTT	AAG	TCG	AAA	ATT	CAA	GAC							
CAA	CTT	AGA	AGG	CTA	TGG	TAG	CTG	TTG	CAA	TTC	AGC	TTT	TAA	GTT	CTG							
					35						40						45					
Lys	Glu	Gly	Ile	Pro	Pro	Asp	Gln	Gln	Arg	Leu	Ile	Phe	Ala	Gly	Lys							
AAG	GAA	GGT	ATC	CCT	CCA	GAT	CAA	CAA	AGA	TTG	ATC	TTT	GCC	GGT	AAG							
TTC	CTT	CCA	TAG	GGA	GGT	CTA	GTT	GTT	TCT	AAC	TAG	AAA	CGG	CCA	TTC							
					50						55						60					
Gln	Leu	Glu	Asp	Gly	Arg	Thr	Leu	Ser	Asp	Tyr	Asn	Ile	Gln	Lys	Glu							
CAG	CTA	GAA	GAC	GGT	AGA	ACG	CTG	TCT	GAT	TAC	AAC	ATT	CAG	AAG	GAG							
GTC	GAT	CTT	CTG	CCA	TCT	TGC	GAC	AGA	CTA	ATG	TTG	TAA	GTC	TTC	CTC							
					65						70						75					
Ser	Thr	Leu	His	Leu	Val	Leu	Arg	Leu	Arg	Gly	Gly	Asp	Ser	Glu	Glu							
TCC	ACC	TTA	CAT	CTT	GTG	CTA	AGG	CTC	CGC	GGT	GGT	GAT	TCT	GAG	GAA							
AGG	TGG	AAT	GTA	GAA	CAC	GAT	TCC	GAG	GCG	CCA	CCA	CTA	AGA	CTC	CTT							
					85						90						95					
Asp	Glu	Glu	His	Thr	Ile	Ile	Thr	Asp	Thr	Glu	Leu	Pro	Pro	Leu	Lys							
GAT	GAA	GAA	CAC	ACA	ATT	ATC	ACA	GAT	ACG	GAG	TTG	CCA	CCA	CTG	AAA							
CTA	CTT	CTT	GTG	TGT	TAA	TAG	TGT	CTA	TGC	CTC	AAC	GGT	GGT	GAC	TTT							
					100						105						110					
Leu	Met	His	Ser	Phe	Cys	Ala	Phe	Lys	Ala	Asp	Asp	Gly	Pro	Cys	Lys							
CTT	ATG	CAT	TCA	TTT	TGT	GCA	TTC	AAG	GCG	GAT	GAT	GGC	CCA	TGT	AAA							
GAA	TAC	GTA	AGT	AAA	ACA	CGT	AAG	TTC	CGC	CTA	CTA	CCG	GGT	ACA	TTT							
					115						120						125					
Ala	Ile	Met	Lys	Arg	Phe	Phe	Phe	Asn	Ile	Phe	Thr	Arg	Gln	Cys	Glu							
GCA	ATC	ATG	AAA	AGA	TTT	TTC	TTC	AAT	ATT	TTC	ACT	CGA	CAG	TGC	GAA							
CGT	TAG	TAC	TTT	TCT	AAA	AAG	AAG	TTA	TAA	AAG	TGA	GCT	GTC	ACG	CTT							
					130						135						140					
Glu	Phe	Ile	Tyr	Gly	Gly	Cys	Glu	Gly	Asn	Gln	Asn	Arg	Phe	Glu	Ser							
GAA	TTT	ATA	TAT	GGG	GGA	TGT	GAA	GGA	AAT	CAG	AAT	CGA	TTT	GAA	AGT							
CTT	AAA	TAT	ATA	CCC	CCT	ACA	CTT	CCT	TTA	GTC	TTA	GCT	AAA	CTT	TCA							
					145						150						155					
Leu	Glu	Glu	Cys	Lys	Lys	Met	Cys	Thr	Arg	Asp	Asn	Ala	Asn	Arg	Ile							
CTG	GAA	GAG	TGC	AAA	AAA	ATG	TGT	ACA	AGA	GAT	AAT	GCA	AAC	AGG	ATT							
GAC	CTT	CTC	ACG	TTT	TTT	TAC	ACA	TGT	TCT	CTA	TTA	CGT	TTG	TCC	TAA							

Figure 4



<u>Lane</u>	<u>Sample</u>
1	MW markers
2	Purified yeast TFPI 3 µg
3	Whole disruptate
4	Supernatant
5	First wash
6	Second wash
7	Insoluble fraction
8	Insoluble fraction 5x load
9	Purified yeast TFPI 3 µg
10	MW markers

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PRODUCTION OF TISSUE FACTOR PATHWAY INHIBITOR

the specification of which (check one) x is attached hereto _____ was filed on August 5, 1994 as Application Serial No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s) Number	Country	Day/Month/Year Filed	Priority Claimed Yes No
--	---------	----------------------	----------------------------

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status Patented, Pending, Abandoned
---------------------------	-------------	--

I hereby declare that all statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Michael A. Innis

Inventor's signature Michael A. Innis Date 5 Aug 94

Residence MORAGA, CALIFORNIA

Citizenship U.S.A.

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Full name of third inventor _____
Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

Full name of fourth inventor _____
Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

Full name of fifth inventor _____
Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

Full name of sixth inventor _____
Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

Full name of seventh inventor _____
Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____